

Inhalation of Diesel Engine Exhaust Affects Spermatogenesis in Growing Male Rats

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We conducted experiments to determine whether diesel engine exhaust affects reproductive endocrine function in growing rats. The rats were assigned to three groups: a group exposed to total diesel engine exhaust containing 5.63 mg/m³ particulate matter, 4.10 ppm nitrogen dioxide, and 8.10 ppm nitrogen oxide; a group exposed to filtered exhaust without particulate matter; and a group exposed to clean air. Dosing experiments were performed for 3 months beginning at birth (6 hr/day for 5 days/week). Serum levels of testosterone and estradiol were significantly higher in animals exposed to total diesel exhaust and filtered exhaust ($p < 0.05$ for each group) as compared to the controls. Follicle-stimulating hormone was significantly decreased in the two groups exposed to diesel exhaust as compared to the control group ($p < 0.05$). Luteinizing hormone was significantly decreased in the total exhaust-exposed group as compared to the control and filtered groups ($p < 0.05$). Although testis weight did not show any significant difference among the groups, sperm production and activity of testicular hyaluronidase were significantly reduced in both exhaust-exposed groups as compared to the control group. Histological examination showed decreased numbers of step 18 and 19 spermatids in stage VI, VII, and VIII tubules in the testes of both diesel exhaust-exposed groups. This study suggests that diesel exhaust stimulates hormonal secretion of the adrenal cortex, depresses gonadotropin-releasing-hormone, and inhibits spermatogenesis in rats. Because these effects were not inhibited by filtration, the gaseous phase of the exhaust appears to be more responsible than particulate matter for disrupting the endocrine system. **Key words:** diesel exhaust, estradiol, follicle-stimulating hormone, hyaluronidase, luteinizing hormone, quantitative morphometry, rats, spermatogenesis, testicular toxicity, testosterone. *Environ Health Perspect* 107:539–544 (1999). [Online 2 June 1999] <http://ehpnet1.niehs.nih.gov/docs/1999/107p539-544watanabe/abstract.html>

Diesel exhaust emission comprises thousands of chemical components, including nitrogen oxide, dioxinlike compounds, and polycyclic aromatic hydrocarbons (1–3). Although it has been hypothesized that such chemicals may contribute directly or indirectly to disorders of the reproductive tract (4–6), there have been few biological studies of endocrine disruption by diesel engine exhaust.

The inhalation of diesel engine exhaust has been reported to increase bone mass in the lumbar vertebrae due to a decrease in bone resorption in growing rats (7). The long-term inhalation of diesel exhaust disturbed the increase of bone mass, changed bone structure, and resulted in fragile bones (8). It is generally agreed that estrogen levels are associated with bone density in females (9,10), and adrenal androgen has also been recognized as a factor in accumulating and maintaining bone density (11–18). Recently, clinical findings (19,20) and experimental investigations using estrogen receptor knock-out (ERKO) mice (21) have indicated that estrogen plays crucial roles in both sexes. ERKO males and females are infertile and show lower bone density by 20–25% than wild-type mice (21). These findings strongly suggest that inhalation of diesel exhaust might affect bone metabolism through changes of reproductive endocrine functions.

The present study was undertaken to determine the effects of diesel exhaust on reproductive endocrine functions by measuring testosterone, estrogen, luteinizing hormone, and follicle-stimulating hormone in male rats exposed to diesel exhaust. Daily sperm production, hyaluronidase activities, and histological changes in the testis were examined as markers of reproductive toxicity. The effects of total exhaust versus control, filtered exhaust versus control, and total exhaust versus filtered exhaust were also compared in order to clarify the type of agent that affects reproductive endocrine functions.

Materials and Methods

Animals. Rats used in the experiment were derived from 24 pregnant female Fischer rats (F344/DuCrj) purchased from Charles River Japan (Kanagawa, Japan). On arrival, they were maintained in a clean room until 3 days before the expected delivery. The clean room was maintained at $24 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ humidity.

On the 19th day of pregnancy, the female rats, six rats for each group, were divided into three groups and placed in separate inhalation chambers. The purpose of transfer from the clean room to the inhalation chamber on the 19th day of pregnancy

before their delivery was to assimilate the mother rats to the new environment and avoid the possibility of cannibalism.

Litter sizes ranged from 9 to 11 pups. The number of animals and the sex ratio of litters in the total group, filtered group, and control group were 76 (male:female = 36:40), 76 (36:40), and 74 (35:39), respectively. The young rats were weaned at 21 days of age. At the same time, the young rats were divided into groups of males and females; male rats were randomly assigned to groups of six housed in a single cage.

The treatment and care of the rats was under an approved protocol of the Animal Care and Use Committee of the Tokyo Metropolitan Research Laboratory of Public Health in a facility approved by the Japan Association for Accreditation of Laboratory Animal Care.

Experimental design. Animals were assigned to three groups: Group 1 was exposed to total diesel engine exhaust (total exhaust); Group 2 was exposed to filtered exhaust without particles (filtered exhaust); and Group 3 was exposed to clean air (control). Each group of animals was maintained in an inhalation chamber (1.6 m³) at $24 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ humidity on a 12 hr light:12 hr dark illumination schedule. The diet was standard rat chow containing 1.03% calcium, 0.70% phosphorus, and 200 IU vitamin D₃/100g (MF, Oriental Yeast Co. Ltd, Tokyo, Japan). All animals were allowed free access to food and water. Exposures began at birth and continued to 3 months of age. The exposure period was 6 hr daily, 5 days/week. Six male rats were selected at random from each group for study at 90 days of age. At the end of the experiment, body weights were measured and blood samples were collected from the abdominal aorta under ether anesthesia. After the animals were killed by exsanguination, testes were removed and weighed.

Generation of diesel exhaust. Diesel engine exhaust was generated by running a 309-cc engine (Model NFAD50; Yanmar

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Diesel Co., Osaka, Japan) at 2,400 revolutions per minute. Exhaust was diluted with clean air in a dilution tunnel and then drawn into the inhalation chamber (5.63 mg/m³ particulate matter, 4.10 ppm nitrogen dioxide, 8.10 ppm nitrogen oxide). For the filtered group, most of the diesel soot particles in whole exhaust were removed by high-efficiency particulate air (HEPA) filtration (ATM 3QA; Nippon Muki Co., Tokyo, Japan). After filtration, 99.9998% of particles larger than 0.05 µm were eliminated. Ventilation was maintained by 15 air exchanges/hour. Concentrations of nitrogen dioxide and nitrogen monoxide were continuously monitored with a chemiluminescent analyzer (Model 8440; Monitor Labs Co., San Diego, CA). Gravimetric measurements of the particulate matter were conducted daily using an automatic beta-ray dust-mass monitor (Model BAM-102; Shibata Scientific Technology Co., Tokyo, Japan). Measurement of particle sizes with a particle fractionating sampler (Andersen Type low pressure impactor LP-20; Tokyo Dylec Co., Tokyo, Japan) confirmed that more than 90% of the particulate matter in the diesel exhaust was smaller than 0.5 µm.

Serum hormonal assay. Serum testosterone and estradiol levels were determined using Enzyme Immunoassay Kits (Cayman Chemical, Ann Arbor, MI). Serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were determined using a rat LH enzyme immunoassay system and a rat FSH enzyme immunoassay system (Amersham, Buckinghamshire, England), respectively.

Daily sperm production. Daily sperm production in the right testis was determined by counting homogenization-resistant spermatids, using the techniques of Sharpe et al. (22,23). Briefly, each right testis was fixed in 10% neutral formalin until it was analyzed for daily sperm production. The testis was weighed again. Two 50-mg portions of testicular tissue were cut, weighed, and homogenized separately in 5 ml of 0.15 M NaCl, 0.05% Triton X-100, using a homogenizer. Homogenization-resistant step 18 and 19 spermatids were counted separately using a hemocytometer in three aliquots of each of the two homogenates per sample, and the mean of the six measurements was calculated. This value was corrected for sample weight and overall testis weight, and transformed to the daily sperm production by dividing by the appropriate time divisor, according to Leblond and Clermont (24).

Hyaluronidase assay. For enzyme assays, "nonanchored" fractions containing nonmembrane-band hyaluronidase and "anchored" fractions containing hyaluronidase, which is present on the plasma

membrane of sperm and enables sperm to penetrate the cumulus barrier, were extracted from each left testis according to the method of Lin et al. (25). The protein contents in the nonanchored and anchored fractions were quantified according to the method of Lowry et al. (26), and the fractions were adjusted to adequate concentrations for the enzyme assay with 0.1 M phosphate buffer, pH 7.0, containing 0.15 M NaCl.

Hyaluronidase activity was measured by quantification of increasing *N*-acetylglucosamine residue at the reducing end of hyaluronic acid, which was liberated by the enzyme. Substrate solution was 1% hyaluronic acid solution. Standard solution was 0.24 µmol/ml *N*-acetylglucosamine solution. Sample solution or phosphate buffer (100 µl) was placed into the tube, which was in ice, and 200 µl of substrate solution or standard solution was added. After mixing thoroughly, the reaction mixture was incubated at 37°C for 10 min. The tubes were then placed in a boiling water bath for 5 min and cooled in ice. After adding 100 µl 5% K₂B₄O₇ · 7H₂O, the tubes were boiled for 7 min and cooled. Next, 1.5 ml 1% *p*-dimethylaminobenzaldehyde acetic acid solution containing 1.25% HCl was added to each tube and the tubes were again incubated at 37°C for 20 min. The absorbance at 585 nm of the supernatant fluid was determined and reducing end *N*-acetylglucosamine was measured. One enzyme unit (ECU) was defined as the liberation of 1 µmol of the reducing end; we also measured *N*-acetylglucosamine residue released from substrate per minute.

Morphologic and immunohistochemical examinations. The right testis from each male rat was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin-eosin

and Periodic Acid Schiff for light microscopy. Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) was performed using a PCNA kit (NCL-PCNA-Paraffin; DAKO, Carpinteria, CA) on serial sections alternating with those used for the histological examinations. Slides included with the kit were used as a positive control.

We performed quantitative evaluation of spermatogenic cells in seminiferous tubules blindly using the simplified morphological method described by Matsui et al. (27). Precise observation of the cycle of spermatogenesis with the morphometric approach is needed when only minimal changes are detected by histopathological examination, and it aids in the investigation of the nature of spermatogenic lesions. Briefly, a total of five seminiferous tubules exhibiting round shape per animal were randomly selected for each stage (stage II-III, V, VII, and XII) of the spermatogenic cycle, and the numbers of seminiferous epithelia were counted. In accordance with the criteria of Leblond and Clermont (24), we were able to distinguish the following spermatogenic cell types: spermatogonia, preleptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes, and round spermatids. The data were expressed as the numbers of spermatogenic cells per Sertoli cell per seminiferous tubule cross section.

Statistical analyses. All reported values were expressed as means ± standard deviations (SD). One-way analysis of variance (ANOVA) followed by Scheffé's test was used among three groups. The statistical significance comparing testis weight, epididymis weight, relative weight, the levels of serum hormones, sperm production, hyaluronidase activity, and the number of spermatocytes between any two groups was

Table 1. Serum testosterone, estradiol, follicle-stimulating hormone, and luteinizing hormone in growing rats.

Hormone	Total exhaust	Filtered exhaust	Control
Testosterone (pg/ml)	1,049 ± 372*	1,065 ± 236 *	604 ± 237
Estradiol (pg/ml)	27 ± 8*	26 ± 12*	15 ± 4
Follicle-stimulating hormone (ng/ml)	208 ± 56*	236 ± 47*	299 ± 48
Luteinizing hormone (ng/ml)	8 ± 1*,**	12 ± 2	12 ± 3

Values are expressed as means ± standard deviations of six rats.

*Different from control; *p* < 0.05. **Different from filtered exhaust; *p* < 0.05.

Table 2. Body weights, organ weights, and relative weights of right testis and epididymis in male rats exposed to diesel exhaust.

Measures	Total exhaust	Filtered exhaust	Control
Body weight (g)	275 ± 19	290 ± 11	279 ± 10
Testis weight (mg)	1,505 ± 62	1,534 ± 48	1,486 ± 56
Relative testis weight (mg/g body weight)	5.49 ± 0.26	5.3 ± 0.14	5.32 ± 0.34
Epididymis weight (mg)	467 ± 17	468 ± 13	470 ± 23
Relative epididymis weight (mg/g body weight)	1.7 ± 0.1	1.61 ± 0.04	1.69 ± 0.1

Values are expressed as means ± standard deviations of six rats.

determined with Student's *t*-test. *p*-Values < 0.05 were considered significant.

Results

Serum levels of reproductive hormones. Serum levels of testosterone and estradiol were significantly higher in animals exposed to total diesel exhaust and filtered exhaust ($p < 0.05$) than in controls (Table 1).

FSH was significantly decreased in the two groups exposed to diesel exhaust as compared to the control group ($p < 0.05$). LH was significantly decreased in the total exhaust exposed-group as compared to the control group ($p < 0.05$). Serum levels of LH were also significantly different between the two groups exposed to diesel exhaust ($p < 0.05$) (Table 1).

Body weights, organ weights, and sperm parameters. The parameters of body weight, right-testis weight, epididymis weight, and relative weight did not show any significant difference among the groups (Table 2).

By external appearance, the testes from the control and exhaust-exposed animals were indistinguishable. Male reproductive organs, including accessory sex organs, developed fully, and no structural malformations were detected in exposed animals.

Sperm production was significantly decreased in both groups exposed to diesel exhaust ($p < 0.01$ and $p < 0.05$ for total and filtered exhaust, respectively) as compared to rats exposed to clean air (Figure 1).

The specific activity of a testicular enzyme associated with postmeiotic spermatogenic cells, anchored hyaluronidase, was lower in the diesel exhaust-exposed rats than in the controls ($p < 0.05$) (Figure 2). The left testis weight and relative testis weight did not show any significant difference among the groups.

Morphologic and immunohistochemical examinations. Histological examination showed decreased numbers of step 18 and step 19 spermatids in stage VI, VII, and VIII tubules and increased numbers of degenerated cells intermediate in development between spermatocytes and terminal stage spermatids in the seminiferous lumen of diesel exhaust-exposed animals. No remarkable histopathological changes were observed in Leydig or Sertoli cells (Figure 3).

Immunohistochemical examination showed an increased number of seminiferous tubules in stage II with decreased numbers of PCNA-positive cells (Figure 4).

Sperm morphological examinations revealed that the numbers of round spermatids in stages II–III, V, and VII were significantly decreased in the diesel exhaust-exposed rats as compared to controls (Figure 5). The numbers of spermatogonia

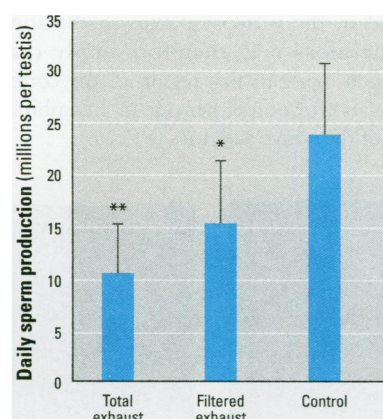


Figure 1. Daily sperm production (means \pm standard deviations) in rats exposed to total diesel exhaust or filtered exhaust or in controls.

* $p < 0.05$ and ** $p < 0.01$, as compared to controls.

and pachytene spermatocytes in stages II–III were also significantly decreased in the diesel exhaust-exposed rats as compared to controls. The numbers of pachytene spermatocytes in stage VII were significantly decreased in the diesel exhaust-exposed rats as compared to the controls ($p < 0.05$ and $p < 0.01$ for total and filtered exhaust, respectively).

In stage XII, the number of spermatogonia of both exhaust-exposed groups was decreased ($p < 0.05$; Figure 5). The numbers of zygotene and pachytene spermatocytes in the filtered diesel exhaust-exposed group were decreased as compared to the control group ($p < 0.01$ and $p < 0.05$ for zygotene and pachytene spermatocytes, respectively). The number of zygotene and pachytene spermatocytes in the filtered diesel exhaust-exposed group was also significantly lower than in the total exhaust exposed-group ($p < 0.01$).

Quantitative evaluation of the populations of Sertoli cells in each stage showed no differences among three groups.

Discussion

This study clearly demonstrated that inhalation of diesel engine exhaust increased serum testosterone and estradiol and reduced serum FSH in growing rats. Spermatogenesis in the testis was defective in both exhaust-exposed groups, although testis weight and the ratio of testis weight to body weight did not differ among the three groups.

Because elimination of the particulate matter by filtration did not seriously influence the results, it appears that the gaseous phase of the exhaust contains agents that are responsible for disruption of reproductive endocrine function. The gaseous phase of diesel engine exhaust includes several agents that may affect endocrine functions. One is nitrogen oxide (28,29). Nitrogen

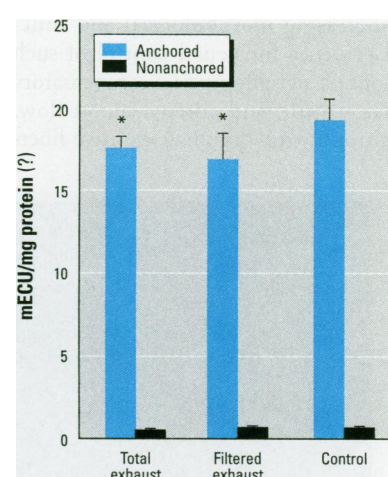


Figure 2. Hyaluronidase activity of anchored and nonanchored fraction (means \pm standard deviations) in testes of rats exposed to total diesel exhaust or filtered exhaust or in control animals. ECU, enzyme unit.

* $p < 0.05$ as compared to controls.

oxide is not only inhaled, but is also synthesized endogenously via a biochemical reaction-dependent process, especially in the sites of inflammation caused by nitrogen dioxide (30–35). Nitrogen oxide is known to be involved in the regulation of endocrine processes such as hypothalamic–pituitary–adrenal and –gonadal axis functions (36–42).

Sperm production and hyaluronidase activity, one of the biochemical markers for testicular toxicity (43–45), were reduced in the diesel exhaust-exposed rats. Serum FSH levels were reduced as well. These elements indicate that testicular function was suppressed by the inhalation of diesel exhaust. However, the inhalation did not cause testicular weight loss. Therefore, increased serum levels of testosterone and estrogen were not derived from reproductive organs stimulated by gonadotropin-releasing hormone in the anterior pituitary, but instead seemed to be induced by increased secretion from the adrenal cortex. Although serum levels of adrenal androgen were not measured directly, there is some evidence that indicates elevated adrenal androgen secretion. The average weight of the adrenal gland in the exhaust-exposed groups was larger than that in the control group in males and females after a 3-month exposure from birth (46). Urinary excretion of 17-ketosteroids, which are among the metabolites of androgens from the adrenal cortex and sexual glands (47,48), was markedly elevated in female rats exposed to diesel exhaust (46).

While the adrenal cortex has traditionally been considered to be principally controlled by adrenocorticotrophin, there is

now increasing morphological and functional evidence for neural control of such functions as steroidogenesis, compensatory adrenal growth, and adrenal blood flow. The nitrogen oxide synthase-positive fibers

found in the varicose plexus in the zona glomerulosa may therefore imply that nitrogen oxide in this region of the cortex exerts its effect on either one or a combination of the above activities (49).

As for adrenal androgens, dehydroepiandrosterone sulfate and dehydroepiandrosterone are not directly associated with the development of reproductive organs and sexual maturity. These adrenal

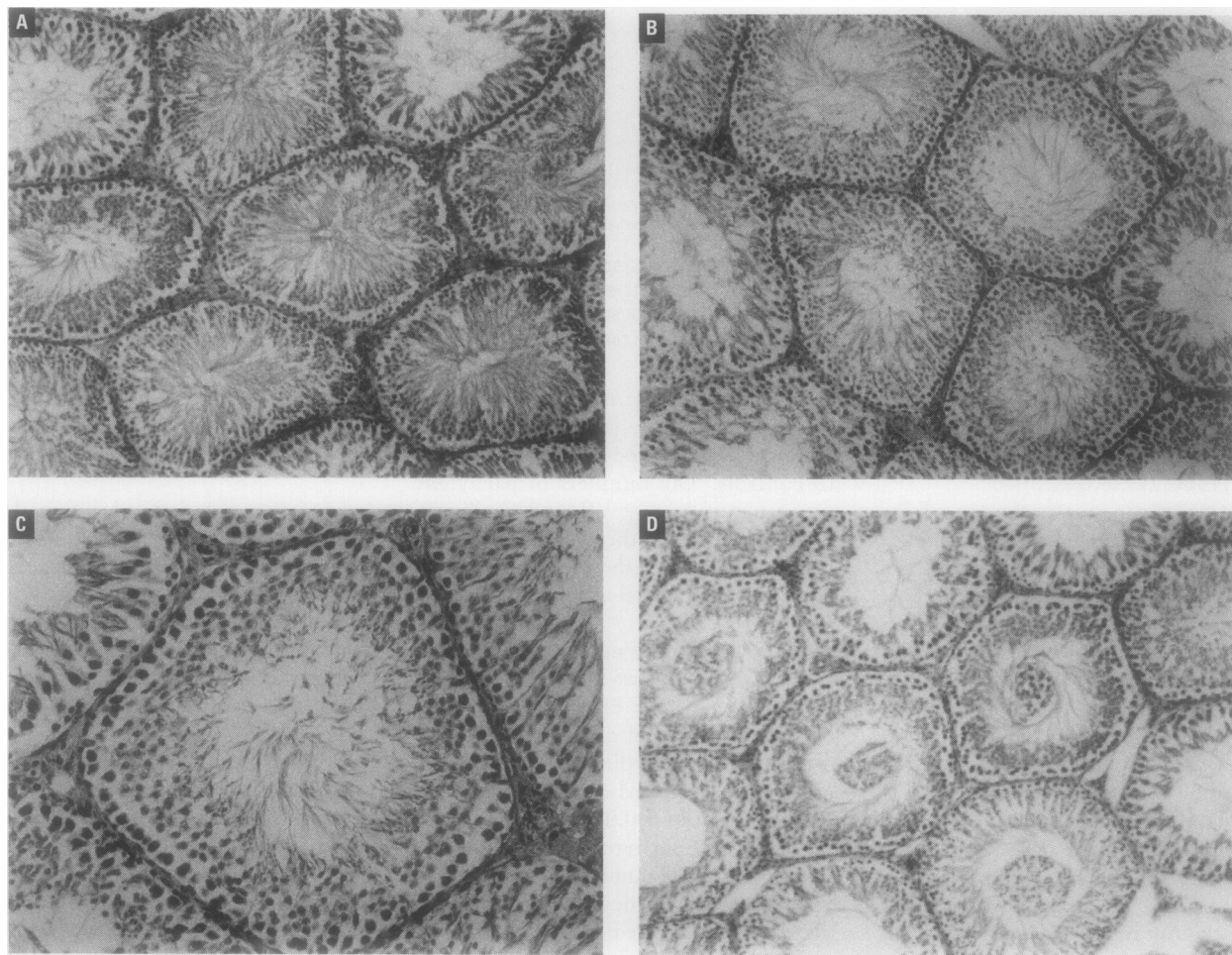


Figure 3. Morphology of seminiferous tubules (stained with hematoxylin and eosin) including different stages. (A) Control tubules ($\times 66$). (B) Seminiferous tubules from total exhaust-exposed animals ($\times 66$); there are decreased numbers of step 18 and 19 spermatids in stages VI, VII, and VIII tubules. (C) Seminiferous tubules from total exhaust-exposed animals ($\times 132$); there are fewer matured spermatids in the lumens of tubules. (D) Seminiferous tubules from total exhaust-exposed animals ($\times 66$); note increased numbers of degenerated cells intermediate in development between spermatocytes and terminal stage spermatids in the seminiferous lumen.

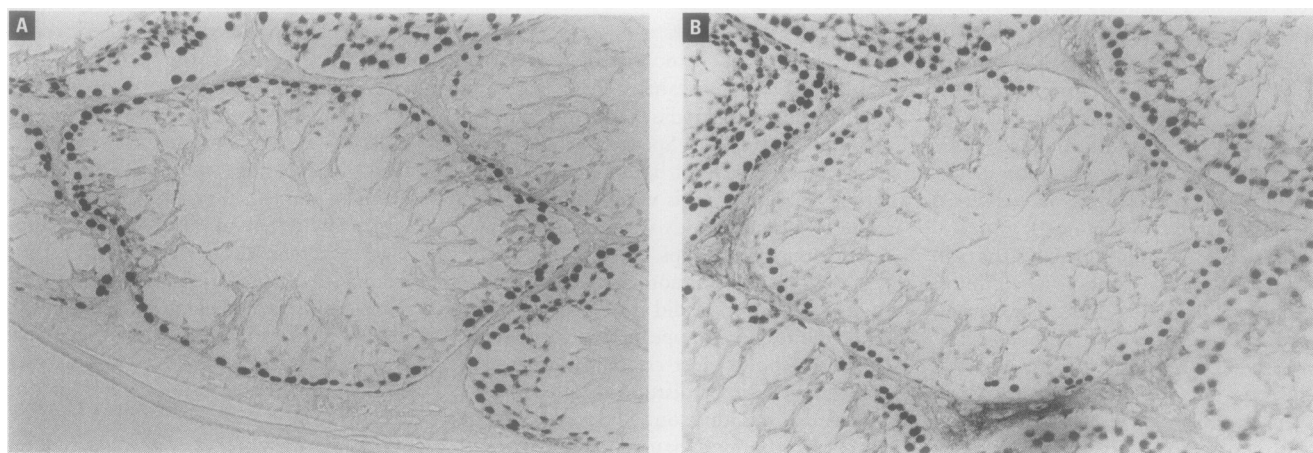


Figure 4. Seminiferous tubules in stage II stained for proliferating cell nuclear antigen (PCNA). (A) From control animals. (B) From animals exposed to total diesel exhaust. Note the decreased number of PCNA-positive cells ($\times 132$).

androgens are converted to testosterone and estrogen by aromatization (50,51). The increased serum levels of testosterone observed in both diesel-exposed groups did not seem to contribute to the growth of the testis. Rather, increased serum levels of testosterone may depress gonadotropic hormones, LH and FSH, that are needed for maintaining normal testicular function (52–54).

It is reasonable to assume that the increased secretion of testosterone and estrogen from the adrenal cortex stimulated directly or indirectly by diesel exhaust caused a negative feedback effect on gonadotropin-releasing hormone in the pituitary gland and depressed spermatogenesis in the testis shortly after sexual maturity.

This study provides evidence that inhaling diesel engine exhaust causes changes in the levels of reproductive hormones and leads to reduction of spermatogenesis in growing rats. This does not necessarily indicate that the altered levels of reproductive hormones detected in these experiments continue in the same way during a long-term exposure (9). The response of reproductive endocrine organs to chemicals depends on the growth stage of the subject animal.

If diesel exhaust were inhaled throughout gestation and neonatal life, male

reproductive function might be impaired by disruption of the development of sex organs because the male reproductive system may be particularly susceptible to toxic insult during the gestation period, as has been observed in the case of dioxin exposure (55–58).

Further studies are necessary to clarify the mechanisms of action of various chemical components in diesel exhaust. The diesel exhaust particulate and gaseous phase concentrations used in this report were 60–100 times higher than those found in the average urban area. Experiments with lower dose exposure will also be necessary to provide reasonable estimates of the human risks of exposure to diesel exhaust.

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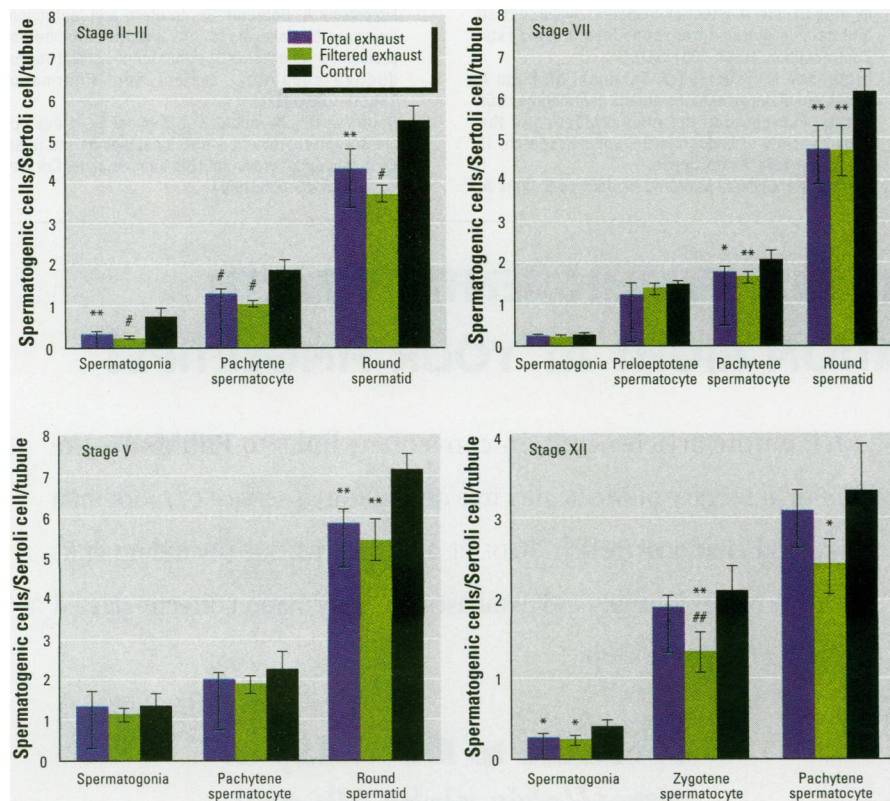
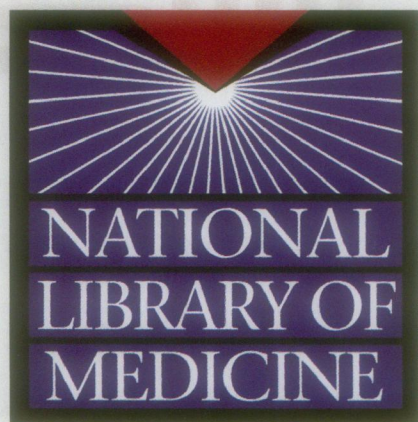


Figure 5. Numbers of spermatogenic cells per Sertoli cell in seminiferous tubules (means \pm standard deviations) of rats exposed to total diesel exhaust or filtered exhaust or in control animals.

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as compared to controls. # $p < 0.01$ as compared to the total exhaust-exposed group.

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